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Occurrence of Dark Septate Endophytes in the Sporophytes of *Christella dentata*

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Abstract.—Mycorrhizal fungi form dynamic symbiotic association in the roots of about 80% of the total terrestrial vascular land plants. Among the pteridophytes, mycorrhizal associations are more frequent in the gametophytes than in the sporophytes. Gametophytes of many ferns and lycophytes exhibit obligate mycotrophy. Despite several benefits derived from the mycorrhizal fungi, very little work has been done surveying the distribution and diversity of arbuscular mychorrhizal (AM) fungi in pteridophytes. We intensified our studies in the roots of sporophytes of *Christella dentata* (Forssk.) Brownsey & Jermy (Thelypteridaceae) to understand the distribution of AM fungi in different parts of the main adventitious root and its lateral branches, along with percent root colonization. We also evaluated spore density of AM fungi in the soil and seasonal variations in the hyphal colonization and formation of vesicles and arbuscules. Root colonization was present in the lateral roots only and not on the main adventitious roots. Percent root colonization also varied in different parts of the lateral roots. The results indicated that root colonization percentage of AM mycorrhizal fungi varies not only by the growth stages of host plants but also by season. Soil spore count was highest in the winter and lowest during the rainy season. In addition to AM fungi, another type of root colonizing fungi, dark septate fungi (DSF) or dark septate endophytes (DSE), has also been recorded in the roots of *C. dentata*. Dark septate fungi with melanized hyphae and microsclerotia were documented.

Key Words.—*Christella dentata*, arbuscular mycorrhizae, dark septate fungus, *Glomus* sp

In addition to arbuscular mycorrhizal fungi (AMF), another type of root colonizing fungi, dark septate fungi (DSF), has been reported within the roots of different seedless vascular plants (Cooper, 1976; Berch and Kendrick, 1982; Dhillion, 1993; Jumpponen and Trappe, 1998). Dark septate fungi are defined by Jumpponen (2001) as conidial or sterile fungi that colonize living plant roots without causing any apparent negative effects. The ecology, taxonomic affinities and host range of these DSF are largely unknown, as is their influence on the host and plant communities (Peterson *et al.*, 2004). However, evidence exists showing that DSE fungi can, under some environmental or experimental conditions, enhance host growth and nutrient uptake, hence functioning in a manner typical of mycorrhizal associations (Mandyam and Jumpponen, 2005). If it is accepted that mycorrhizal fungi cause host responses mainly mutualistic with long-term fitness effects, the DSE association should be included when the diversity of mycorrhizal symbioses and responses are considered. Including DSE in mycorrhizal studies would yield valuable information about the importance and frequency of these root colonizers (Jumpponen and Trappe, 1998). Hence, in the present study, an attempt has been made to investigate

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the association of DSE and AM in the roots of a common pteridophyte, *Christella dentata* (Forssk.) Brownsey & Jermy to understand the diversity of fungal root endosymbionts in this plant.

Materials and Methods

Christella dentata, a common terrestrial fern in the plains of India, grows in broad ecological conditions. Ten young and 10 mature sporophytes were collected from Onda, Bankura district of West Bengal, India at latitude 23°14′N and longitude 87°14′E in each of the three different seasons: winter (November to February), late spring to summer (March to May) and rainy season or monsoon (June to October) for two consecutive years: 2008 and 2009.

Roots were collected by digging and uprooting the whole plant along with the rhizospheric (root region) soil. Root samples were thoroughly washed in running tap water and rootlets were selected. The average root length of the main adventitious root system was measured to be 10.00–11.33 cm. Then, the root system was grouped into two categories: main roots and lateral roots. Each of the root categories was divided into three equal portions: the distal, the middle and the basal portions to determine the variation of mycorrhizal colonization in different regions of the root. Each was cut into small pieces (1 cm in length) and fixed in formaldehyde acetic acid solution (Johansen, 1940) and stored at 4°C.

About 10 g of soil were collected from the rhizosphere of *C. dentata* by digging the soil up to a depth of 15 cm. The soil that adhered to the uprooted roots was separated and added to the soil collection. The total soil collected from around each sporophytic plant of *C. dentata* was kept in separate polythene packets, labeled and stored at 4°C until analysis. Three such soil samples were collected in each season.

Root samples were stained following the method of Phillips and Hayman (1970). For each specimen, 100 root pieces from each of the samples were thoroughly washed in water and boiled at 95°C for 1 hour in 10% KOH. The root segments were washed in distilled water, acidified with 1(N) HCl for 5 minutes and stained in 0.05% trypan blue in acidic glycerol for overnight. The excess colorant was removed by washing with 10% glycerol. Root segments were mounted on slides in acetic acid and glycerol (1:1 V/V) and the edges of the cover slips were sealed with DPX and observed under microscope (Leica, model no. DMLB).

The mycorrhiza type present in the root samples was determined according to the method of Harley and Smith (1983). Arbuscular mycorrhizae were examined in the root samples as percent mycorrhizal association and was calculated as follows:

$$\%\ \text{Mycorrhizal association} = \frac{\text{No. of mycorrhizae associated segments}}{\text{Total No. of segments scored}} \times 100$$

Ten grams of soil was dissolved in 100 ml of distilled water in a conical flask. The conical flask was shaken for 30 min and then kept in undisturbed condition for 30 min. The soil particles precipitated and the spores floated on the surface of the liquid. Mycorrhizal spores were obtained by wet sieving and decanting technique as followed by Gerdemann and Nicolson (1963).

The soil solution was passed through 350, 300, 53 and 45 μm sieves and the spores were collected from the residue deposited on the sieves. For this, residue present on the sieve was dissolved first in distilled water and then filtered with filter paper. The residue present in the filter paper was taken and mounted on a slide in lactophenol and cotton blue and was examined under a microscope (Leica, model no. DMLB). The decantant was filtered through a filter paper with grid lines. The filter paper was then spread on a glass plate under a dissecting microscope and spores were counted and expressed as spores per 100 g of dry soil.

The prepared root samples (trypan blue stained) were also examined for septate hyphae and microsclerotia corresponding to DSE under a Leica microscope. The mature microsclerotia were allowed to germinate and grow in different media *viz.* potato dextrose agar medium, Saberoud medium and malt agar medium. The roots containing mature microsclerotia were surface sterilized and put in potato dextrose agar medium at pH 6.5 and incubated for 30 days for germination of the microsclerotia and to develop the fresh culture of the dark septate endophytic fungus.

Results

The root samples showed the presence of DSE. The septation of the dark septate endophytic fungal hyphae was very prominent and visible even with a very light stain of trypan blue (Fig. 1A). The colonization percentage of DSE was highest in winter to spring and lowest in the rainy season (Table 1). The DSE with microsclerotia (Fig. 1B, C) of different shapes and sizes were highly melanized. The immature microsclerotia (Fig. 1B) took the stain of trypan blue and appeared light blue in color and with maturity the cell wall became thicker and the degree of melanization increased. The microsclerotia were without definite shape, often grew together with the mycelium and remained embedded in the mycelium, bound together with the mycelial strands. Mature microsclerotia (Fig. 1C) did not show well-developed zones of tissue. They were made up of central part made up of pseudoparenchymatous tissue but the hyphal nature exists. Towards the outside of the microsclerotia the hyphae were more loosely arranged.

Upon culturing, branched, dark-colored septate hyphae of the DSE developed, showing small constrictions at branch points. The pure culture of the DSE developed after 30 days of incubation and it was observed that the potato dextrose agar (PDA) medium was most suitable at an optimum pH of 6.5. In culture, microsclerotia developed, the sclerotial initials were found to arise by branching and septation of hyphae. The cells became barrel shaped and considerably wider than the vegetative hyphae, dark brown to reddish – brown in color and was identified as *Rhizoctonia* spp.

Arbuscular mycorrhizae were found in all the lateral root samples studied (Table 1). Both arbuscules (Fig. 2A & B) and vesicles (Fig. 2E) were seen in the roots of *C. dentata*. The number of vesicles was less than the number of arbuscules. The vesicles were large, measuring 38 to 56 μm in diameter, oval to round in shape.

The aseptate hyphae of arbuscular mycorrhizal fungi grew parallel to each other. As the roots were grouped into two categories, main roots and lateral

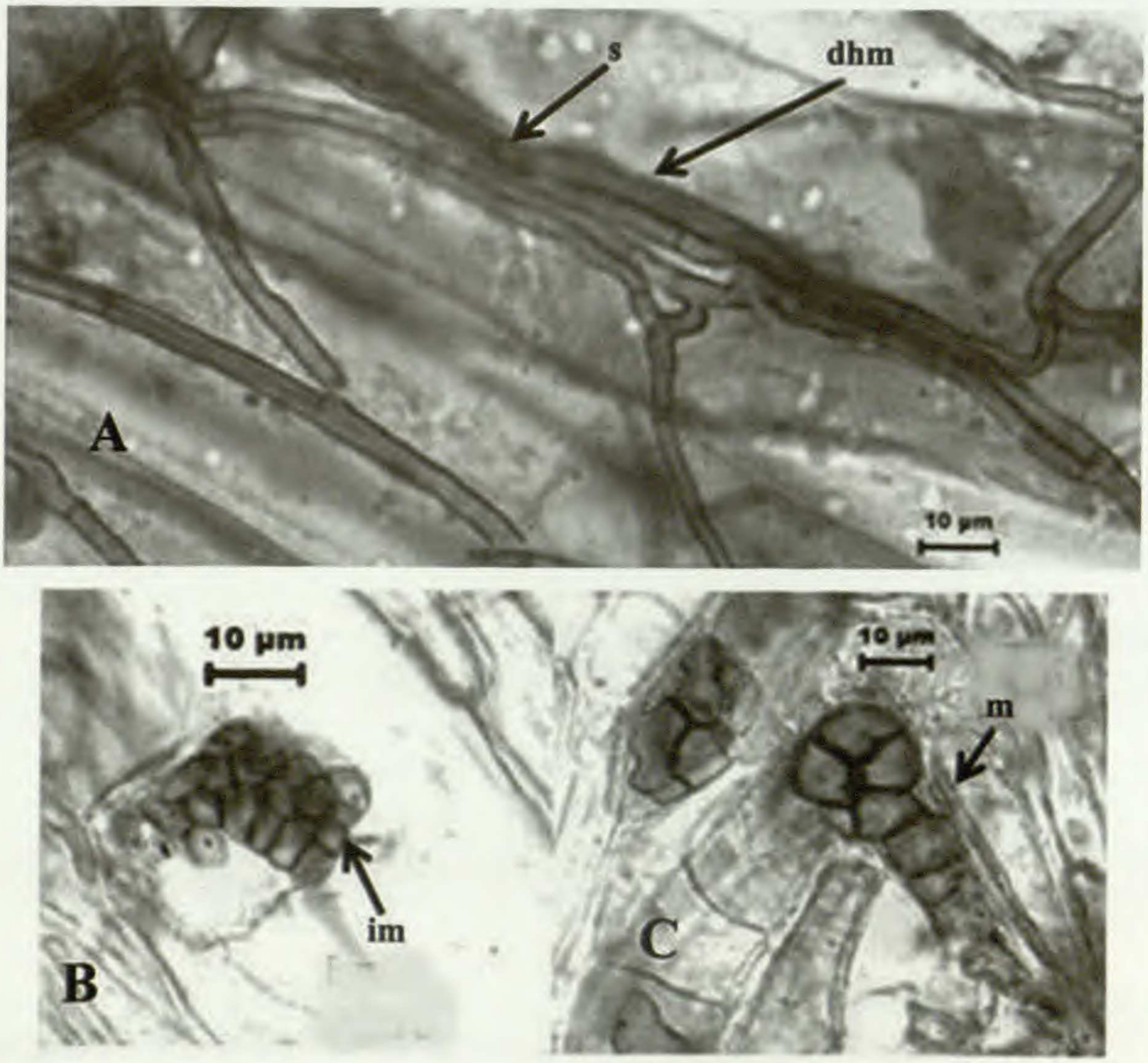

FIG. 1. (A–C) DSE hyphae and microsclerotia within *Christella dentata* A. Septate DSE melanized hyphae B. Immature microsclerotia C. Mature microsclerotia.

roots, they were studied separately and it was evident that the main roots in all the cases showed no mycorrhizal colonization. In contrast, the lateral roots in almost all the cases exhibited mycorrhizal colonization. Percentage of colonization was higher in mature lateral roots (24 to 58.5%) than in young lateral roots (11 to 29%). However, it was noticed that in young plants, the distal portions of the lateral roots had greater mycorrhizal colonization whereas, in mature plants, the basal portions of the lateral roots showed more hyphae and arbuscules. The number of arbuscules and the colonization of aseptate mycorrhizal hyphae was highest in winter and lowest in the rainy season (Table 1). No vesicles were found in young plant roots. Only the mature plant roots possessed vesicles (Fig. 2E) in very low frequency (0.1 to 0.7%).

The root samples of *C. dentata* presented the *Paris*- type of AM colonization (Fig. 2A & B) as the arbuscules, vesicles and intraradical, non-septate hyphae of mycorrhizal fungi were intracellular.

TABLE 1. Colonization of vesicular arbuscular mycorrhizae and dark septate fungi in roots of *Christella dentata*.

			Mycorrhizal colonization				DSF colonization	
Plant growth stage	Seasonof collection	Portion of lateral root	Percentage of hyphae	Percentage of vesicle	Percentage of arbuscle	Percent root colonization*	Presence or absence of hyphae	Presence or absence of microsclerotia
MATURE PLANT	Winter	Tip	18	0.7	36.3	55 ± 1.11	+	−
		Middle	3	0	40	43 ± 1.32	+	+
		Basal	3.5	0	55	58.5 ± 1.10	+	+
	Late spring to summer	Tip	15	0	30	45 ± 0.89	−	+
		Middle	5	0	25.5	30.5 ± 0.90	−	+
		Basal	5	0.1	38.5	43.6 ± 1.22	+	+
	Rainy	Tip	5	0	20	25 ± 1.50	−	−
		Middle	3	0	21	24 ± 1.45	+	−
		Basal	1.5	0	25	26.5 ± 1.00	+	−
YOUNG PLANT	Winter	Tip	19	0	10	29 ± 0.75	+	−
		Middle	18.5	0	9.1	17.6 ± 0.87	+	−
		Basal	20	0	9	29 ± 0.88	+	−
	Late spring to summer	Tip	20	0	7	27 ± 0.65	−	−
		Middle	21	0	3.5	24.5 ± 0.80	−	−
		Basal	18	0	5.5	13.5 ± 1.40	−	−
	Rainy	Tip	10	0	2	12 ± 1.33	−	−
		Middle	10	0	2.5	12.5 ± 1.22	−	−
		Basal	9	0	2	11 ± 0.95	+	−

*Data are the mean values of ten replicates

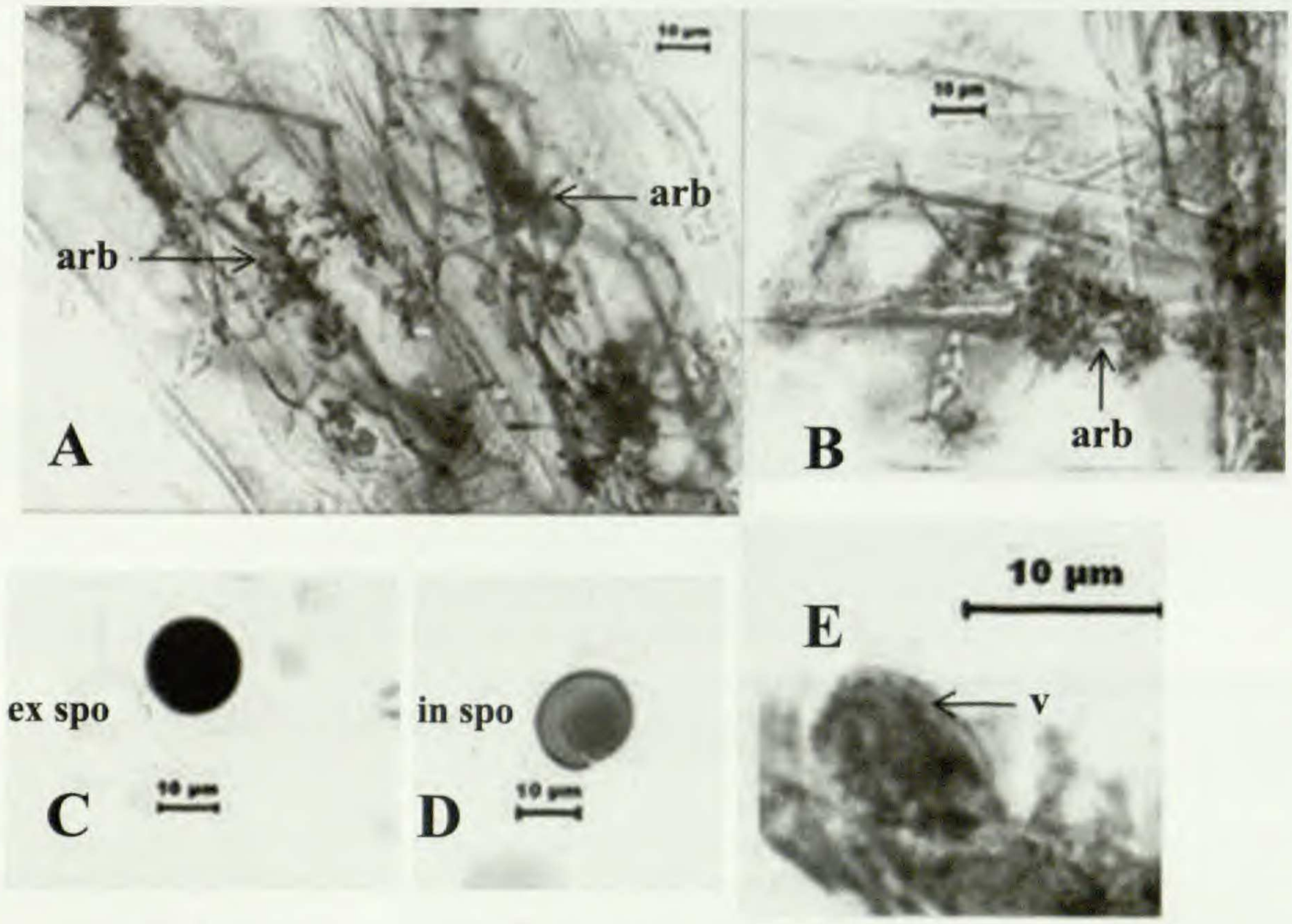

FIG. 2. (A–E) Mycorrhizal arbuscules, vesicle and extra radical spores within roots of *Christella dentata*. A. Arbuscules within *Christella dentata*. B. Arbuscules within *Christella dentata*. C–D. Extra radical spores E. Vesicle within *Christella dentata*.

Spore (Fig. 2C, D) counts per 100 g of soil showed 550 ± 10.44 spores in the winter season, 370 ± 9.89 spores in the late spring to summer, and 210 ± 11.30 spores in the rainy season. The AM fungus has been identified as *Glomus* sp.

DISCUSSION

In this paper, we report occurrence of *Glomus* sp. as arbuscular mycorrhizal fungus in the lateral roots of *Christella dentata* along with dark septate endophytes (DSE), which has been identified as *Rhizoctonia* sp. The arbuscular mycorrhizal fungus *Glomus* was characterized by the formation of large swollen vesicles, well developed arbuscules and extraradical round spores. Though *Glomus* and *Sclerocystis* have been reported earlier by Muthukumar and Udaiyan (2000) as AM in *Christella dentata*, to the best of our knowledge this is the first report of presence of DSE in *Christella dentata* along with microsclerotia.

Mycorrhizal colonization pattern was found to be of *Paris*-type in *Christella dentata* (Fig. 2 A–C). This finding is in conformity with the observations of other studies where this morphotype has been found in other pteridophytes (Zhang *et al.*, 2004; Dickson, 2004; Zubek *et al.*, 2010) and the percent root colonization by AMF varies with the host plants and the habitats have been shown by a number of authors (e.g., Muthukumar and Udaiyan, 2000; Zubek *et al.*, 2010).

Zubek *et al.* (2010) recorded highest AM colonization (M=78%) and arbuscule richness (A = 76%) in *Thelypteris patens* (Sw.) Small and lowest AM colonization and arbuscule abundance in *Sticherus underwoodianus* (Maxon) Nakai (M= 4%, A = 3%). Muthukumar and Udaiyan (2000) showed the intensity of mycorrhizal colonization was significantly influenced by the type of substrate in which the plants grow. Pteridophytes growing in soil (terricolous) had the highest mean colonization levels followed by lithophytic and epiphytic species. As we have studied only one terrestrial member, we are not in a position to compare the result of mycorrhizal colonization in different habitats. However, we have noted that percentage of AM colonization varies not only on the growth stages (young / mature) of host plants but also with the seasonal variations of the year. We found the highest mycorrhizal colonization (58.5 ± 1.10) and spores and arbuscule formations in *Christella dentata* in the winter months and the lowest in the rainy season (Table 1). No vesicles were observed in the rainy season. In summer, there was much less vesicle formation (0.1 0 ± 0.01) than in winter (0.7 ± 0.1). Mycorrhizal colonization was moderate in the summer months. In young lateral roots mycorrhizal colonization percentage was always less than that of the mature roots (Table 1).

It is assumed that the vesicles, being the storage organs of the AM, are generally produced at comparatively later stages of growth (Powell and Bagyaraj, 1984). Bajwa *et al.* (2001), while surveying AM association in wetland plants, noticed that vesicular infection, in general, started in spring and reached its maximum in summer, autumn or winter depending upon the host species. Another possible reason for the disparity in the pattern of AM development may correspond to variations in stages of completion of life cycles of various AM species involved in forming this association. In a survey on AM association in different vascular plants it was found that colonization remained stable during spring to summer and maximum colonization has been observed during winter (Bajwa *et al.*, 2001). The spore population in association with dicotyledonous species remained consistently high except for a decline in autumn. These results corroborate our present findings on *C. dentata* where AM colonization percentage in the roots and spore number in the soil was highest in winter.

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